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(54) Title: A SYSTEM FOR MINIMIZING BACKGROUND FLUORESCENCE IN FLUORESCENCE IMAGING APPLICATIONS USING NEUTRAL DENSITY FILTER MATERIAL SUBSTRATES

(57) Abstract: An invention is disclosed that is comprised of neutral density filter material substrates for fluorescent imaging applications, reducing background fluorescence in the acquisition, compilation, and analysis of fluorescent images.

A SYSTEM FOR MINIMIZING BACKGROUND FLUORESCENCE IN FLUORESCENCE IMAGING APPLICATIONS USING NEUTRAL DENSITY FILTER MATERIAL SUBSTRATES

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority based on U.S. provisional patent application no. 60/159,169 filed October 13, 1999.

FIELD OF THE INVENTION

This invention relates to imaging systems. In particular, this invention relates to imaging systems where reduction of background fluorescence is desired in the acquisition, compilation, and analysis of fluorescent images.

BACKGROUND OF THE INVENTION

The Invention described herein is used for the acquisition, compilation, and analysis of images of fluorescently labeled samples of different types. In an effort to keep up with increasing demands, researchers continue to seek faster, more efficient and reliable data acquisition equipment. By way of example, laser scanning systems are a type of system that has been developed to advance research and improve productivity. Such imaging systems are commonly used to capture imaging information from fluorescently labeled materials.

Many areas of scientific investigation require the use of imaging equipment. As only one example, in the field of genomics research, imaging equipment is used to acquire, compile, and analyze the results from testing of deoxyribonucleic acid ("DNA") "microarrays" (also know as "gene chip arrays", "biochips", and other designations).

Microarrays are prepared as a means to match known and unknown DNA samples based on hybridization principles, for example, to identify gene sequences or to determine gene expression levels. In one method, microarrays can be made by "spotting" collections of suspended, purified DNAs onto a substrate. In a typical production method, a microarray robot places drops of individual DNA types onto a substrate, such as a glass slide, in a grid design. The grid may contain thousands of DNA spots of different base pair sequences that are fixed to the substrate. cDNA "probes" are then tested by hybridizing them to the prepared DNA microarray. If an individual cDNA probe is complementary to the sequence

of DNA on a given spot, the cDNA will hybridize to the spot and the hybridization may be detected by its fluorescence. In this manner, each spot in the microarray may act to assay the presence of a different cDNA.

After the cDNA probes have hybridized to the microarray and any free probe has been removed, the microarray may be scanned to evaluate the comparative binding levels of individual probes. cDNA probes hybridized to DNA spots in the microarray may be detected through the use of different colored fluorophores or dyes that emit light at differential, characteristic wavelengths when excited by an illumination source. Microarray spots with more bound probe will fluoresce more intensely.

Current laser scanning systems collect excitation laser light that is reflected from the slide surface along with fluorescence down the emission beam path. The emitted light is captured by a detector, such as a charge-coupled device ("CCD") or a photomultiplier tube ("PMT"), which records its intensity. The recorded data is stored or processed for further analysis. The detector for fluorescence emitted from the microarray may be sensitive to the emission wavelength but filters out the excitation wavelength; in this way, the fluorescent emission of interest can be separated from the excitation light scattered off the substrate.

Background fluorescence is a cause of error in most, if not all, fluorescence imaging applications. The materials used as substrates may have a pronounced effect on the quality of data acquired from an imaged sample. Typical white glass slides used as substrates may contain contaminants that fluoresce when exposed to an excitation source, or they may produce ghost images. Sample observation methods, such as the use of cover slips and mounting media, may result in focusing error or background contamination in some applications, such as confocal techniques. High background levels may be especially destructive to quantitative applications, such as differential expression analysis using DNA biochips. Software methods to minimize background can and do function well, but often at the expense of other data acquisition criteria, such as dynamic range.

Based upon the aforementioned limitations, there exists a need in the industry for an imaging system that can collect the desired imaging data in an efficient and reliable manner while reducing background.

SUMMARY OF THE INVENTION

The present Invention meets the existing need by reducing background in the scanned image in order to better determine intensity thresholds with more uniform detection efficiency across a scanned sample. The present Invention employs neutral density filter materials as a substrate on which fluorescent material is deposited for analysis. Neutral density filter materials show minimal fluorescence properties and appear less fluorescent than other types of slides typically made for fluorescent imaging applications. The neutral density filter material substrates of the present Invention absorb scattered excitation light that can add to image background and absorb fluorescence generated inside the substrate from the excitation light. The result is that backgrounds are reduced to very small or barely detectable levels, especially when compared to the low-copy message fluorescence seen in certain types of imaged samples.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the use of the present Invention in reducing fluorescent background from contaminants in or on the bottom of a substrate.

Figure 2 shows the use of confocal techniques in imaging a sample on a typical glass substrate.

Figure 3 shows the effect of a displaced typical glass substrate in a confocal application.

Figure 4 shows the use of the present Invention in a nonfocal application where the imaged sample is displaced.

Figure 5 shows the effect of sample observation techniques, such as cover slips and mounting media, in a confocal application.

Figure 6 shows the effect of a tilted coverslip over mounting media in a confocal application.

Figure 7 shows the generation of ghost images in an application using a typical glass substrate.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the present Invention, neutral density filter materials are made into substrates for the observation or imaging of samples in any fluorescent imaging application, including without limitation, automated imaging systems and fluorescence microscopy. As used herein, neutral density filter ("NDF") materials means NDF glass (for example, without limitation, Schott NG1 or Hoya NDF glass) of all types known to those of ordinary skill in the art and any other light absorbing glass. NDF materials show minimal fluorescence properties and appear less fluorescent than the water-white glass from which slides are typically made for fluorescent applications.

By way of example only, minimal fluorescence is an important quality required of genechip substrates for extraction of the small fluorescent signals of low-copy messages from the fluorescent background of the substrate. The NDF material substrates of the present Invention not only absorb scattered excitation light that can add to the genechip image background, but also absorb fluorescence generated inside the substrate from the excitation light. The result is that backgrounds are reduced to very small or barely detectable levels. The background reduction is especially dramatic for shorter wavelength excitation light (488 and 532 nm). This reduction in background improves the sensitivity of the measurement of low, medium, and high-copy messages, and can make the difference between measuring and not measuring the lowest-level messages.

The NDF material used in the present invention may be of any suitable color, by way of examples only, varying shades of black or gray. In the present Invention, substrates may be made in whole or in part of NDF material, and may be prepared in any thickness, width or height appropriate for use in the desired fluorescent imaging application or equipment. By way of example, and without limitation, the NDF material substrate may be prepared in dimensions typical of glass slides known to those of ordinary skill (for example, a rectangle approximately one (1) inch by three (3) inches and one (1) mm thick), or of any other suitable dimensions.

Any sample suitable for fluorescent imaging, including without limitation, DNA, RNA, protein, or biological tissue samples, may be disposed for imaging on the NDF substrate of the present Invention. The NDF substrate may be coated with silane, poly-l-lysine, or another appropriate coating for optimal sample attachment, for example, for

nucleotide attachment. To give NDF slides better surface properties for use as gene chips, vapor deposited silanation by processes known to those of ordinary skill in the art may be used instead of the more usual liquid-based protocols. For consistent deposition of droplets of DNA solution onto substrates, for example, genechip substrates, the wetting properties should be uniform across the entire surface of the slide. More wettable regions remove more fluid from the fluid handling device and produce larger, brighter spots, while less wettable regions produce compact spots which contain less material. This makes automatic image analysis more difficult, and reduces the validity of quantitative comparisons between spots. The substrate surface may also be prepared with suitable chemical properties that are known to those of ordinary skill in the art to allow for covalent binding of DNA, such that it does not wash off during hybridization. Both requirements are met by vapor deposition of silane directly onto the substrate slides.

The NDF material substrates in the present Invention eliminate fluorescence from contaminants on the back of the slide and particles inside the slide. As an example of a preferred embodiment of the present Invention, in Figure 1, a sample comprised of fluorescently label DNA (1) is disposed on an NDF substrate (2). Excitation light (3) from a light source is transmitted to the sample (1) in a viewing area (4) by appropriate means, for example and without limitation, a dichroic beam splitter (5). Emission light (6) from the sample (1) is transmitted to a detector (7). However, the NDF material of the substrate (2) absorbs excitation light and fluorescence from contaminants (8) in the substrate. Thus, as shown in Figure 2, in one embodiment, the Invention replicates confocal behavior of an imaging system 1) without a confocal pinhole (9) used to reject some background emissions; and 2) without the shallow depth of field (10) and precise axial alignment required in confocal imaging.

Moreover, as shown in Figure 3, the shallow depth of field (10) can make automatic slide handling in a confocal instrument difficult to achieve reliably when the substrate is not properly aligned, for example, when it is tilted (12) in the viewing area. As shown in Figure 4, the NDF substrate (2) allows for the use of a non-confocal imaging approach, with a larger depth-of-field (13) that can accommodate some substrate nonalignment with background rejection similar to confocal imaging.

The NDF substrate of the present Invention can be used with a confocal imager and will give lower backgrounds because of the absorptive properties of the NDF material in the confocal section.

The NDF slide can be used with a non-confocal imager, with or without a coverslip and low-fluorescence mounting medium to enhance the lifetime of the fluorescence dyes on the slide. As one example only, an appropriate mounting medium (such as DPX) can extend the lifetime of a Cy5-stained slide by a factor of 10 or more. Mounting mediums and coverslips are difficult to use with a confocal scanner and may result in unreliable quantitation results (see e.g. Figure 5). The confocal section shifts axially with the refractive index change from introduction of a mounting medium under a cover slip (compare (14) and (15) in Figure 5), causing signal rejection and requiring refocusing of the objective. Use of the NDF substrate of the present Invention in a nonconfocal instrument tolerates this focus error with no decrease in fluorescence signal. Depth of focus is deep enough to accommodate the added thickness of a glass cover slip on top of the sample and allow for some variation in the height at which the sample is presented to the imaging system.

In addition, as shown in Figure 6, small differences in mounting medium thickness from one side of the slide to the other can displace the focus and confocal section (16) on one side of the slide, resulting in uneven detection of fluorescence across the field of view. This problem requires an increase in complexity of a confocal scanning mechanism, for example, requiring adjustment of the objective focus during a scan through an "autofocus" feature. In one embodiment of the present invention, a combination of non-confocal imaging means and an NDF substrate, with a larger depth of view, tolerates this error with no autofocus feature.

As shown in the example of Figure 7, the NDF substrate of the present Invention prevents generation of ghost images from reflections off the back of the slide. Ghost images are created when excitation light, for example, laser light, reflect off the back of the slide and excite off-axis fluorescence (17) from objects that are not at the on-axis focus (18) of the objective. The NDF substrate of the present Invention absorbs the excitation light before it can reflect off the back of the slide, thus reducing or eliminating ghost images. It also prevents imaging errors near the edge of the slides produced by funneling of

the excitation light to the edge of the slide. This funneling is caused by multiple reflections of the excitation light from the top and bottom surfaces of the clear slide.

Preferred embodiments of the present Invention have been disclosed. A person of ordinary skill in the art would realize, however, that certain modifications would come within the teachings of this Invention, and the following claims should be studied to determine the true scope and content of the invention. In addition, the systems and methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are described herein. It will be apparent to the artisan that other embodiments exist that do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

CLAIMS

What is claimed is:

1. A system for imaging one or more samples, comprising:

   a. a source of excitation light;

   b. a viewing area;

   c. a labeled sample disposed on a substrate in the viewing area, the substrate comprised of neutral density filter material;

   d. means for transmitting the excitation light to the sample in the viewing area;

   e. a detector of emission light from the sample; and

   f. means for transmitting emission light from the sample exposed to the excitation light to the detector.

2. The system of Claim 1, where said neutral density filter material is comprised of light absorbing glass.

3. The system of Claim 1, wherein said substrate is comprised of black neutral density filter material.

4. The system of Claim 1, wherein said substrate is comprised of gray neutral density filter material.

5. The system of Claim 1, where said source of excitation light is comprised of a laser or an arc lamp.

6. The system of Claim 1, wherein said means of transmitting emission light is comprised of confocal means.

7. The system of Claim 1, wherein said means for transmitting emission light is comprised of nonfocal means.

8. The system of Claim 1, wherein said sample is comprised of one or more nucleic acid molecules.

9. The system of Claim 8, wherein one or more nucleic acid molecule is fluorescently labeled.

10. The system of Claim 1, wherein said sample is comprised of one or more protein molecules.

11. The system of Claim 10, wherein one or more protein molecule is fluorescently labeled.

12. The system of Claim 1, wherein said substrate is coated before disposition of said sample with a coating material.

13. The system of Claim 12, wherein said coating material is comprised of silane or poly-1-lysine.

14. A method for imaging one or more samples, comprising the steps of:

a. providing a source of excitation light;

b. providing a viewing area;

c. providing a sample disposed on a substrate in the viewing area, said substrate comprised of neutral density filter material;

d. providing means for transmitting the excitation light to the sample in the viewing area;

e. providing a detector of emission light from the sample; and

f. providing means for transmitting emission light from the sample exposed to the excitation light to the detector.

15. The method of Claim 14, where said neutral density filter material is comprised of light absorbing glass.

16. The method of claim 14, wherein said substrate is comprised of black neutral density filter material.

17. The method of claim 14, wherein said substrate is comprised of gray neutral density filter material.

18. The method of Claim 14, where said source of excitation light is comprised of a laser or an arc lamp.

19. The method of Claim 14, wherein said means of transmitting emission light is comprised of confocal means.

20. The method of Claim 14, wherein said means for transmitting emission light is comprised of nonfocal means.

21. The method of Claim 14, wherein said sample is comprised of one or more nucleic acid molecules.

22. The method of Claim 21, wherein one or more nucleic acid molecule is fluorescently labeled.

23. The method of Claim 14, wherein said sample is comprised of one or more protein molecules.

24. The method of Claim 23, wherein one or more protein molecule is fluorescently labeled.

25. The method of Claim 14, wherein said substrate is coated before disposition of said sample with a coating material.

26. The method of Claim 25, wherein said coating material is comprised of silane or poly-1-lysine.

27. A substrate comprised of neutral density filter material for use in one or more fluorescent imaging applications.

Figure 1.

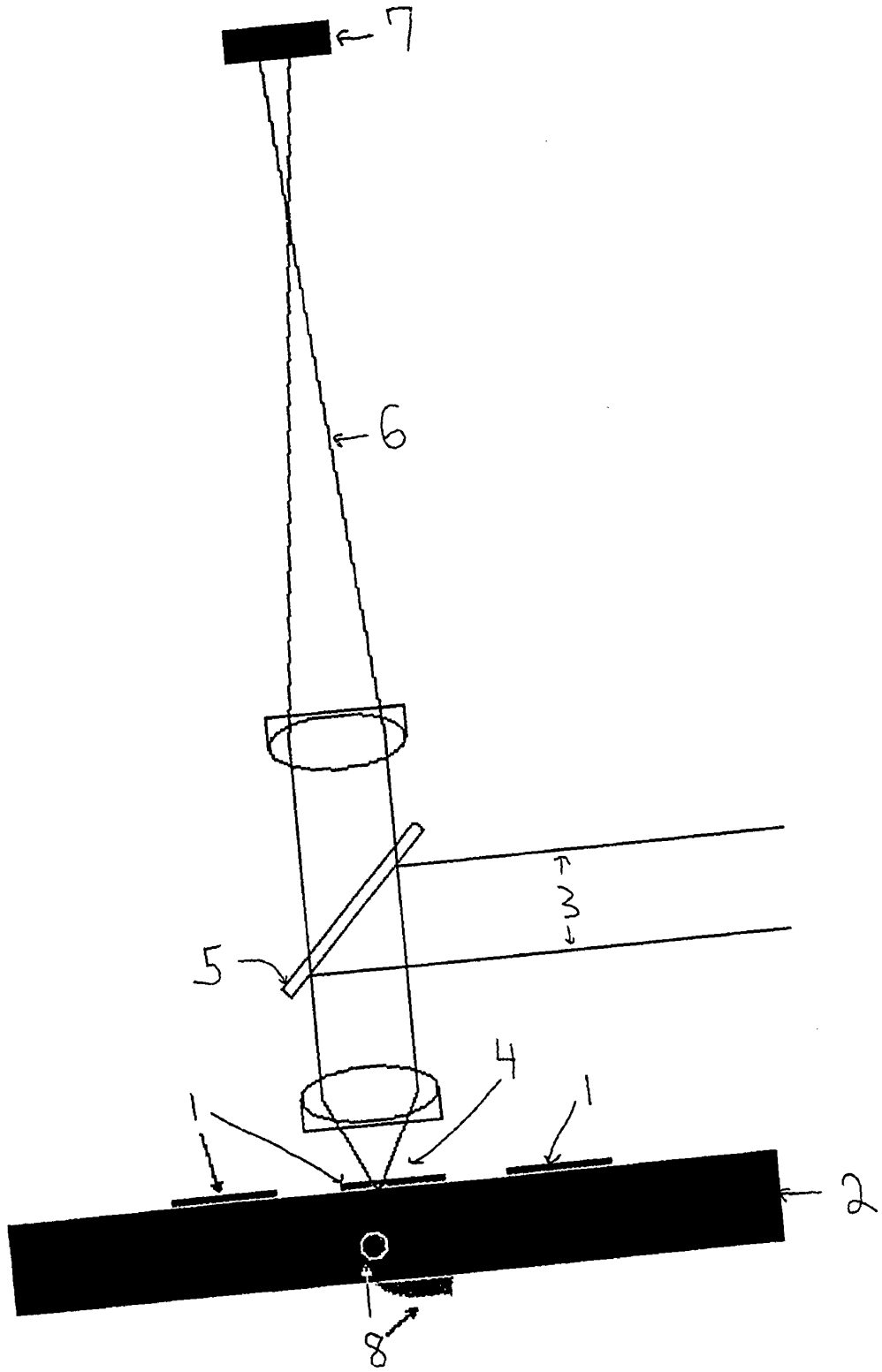


Figure 2.

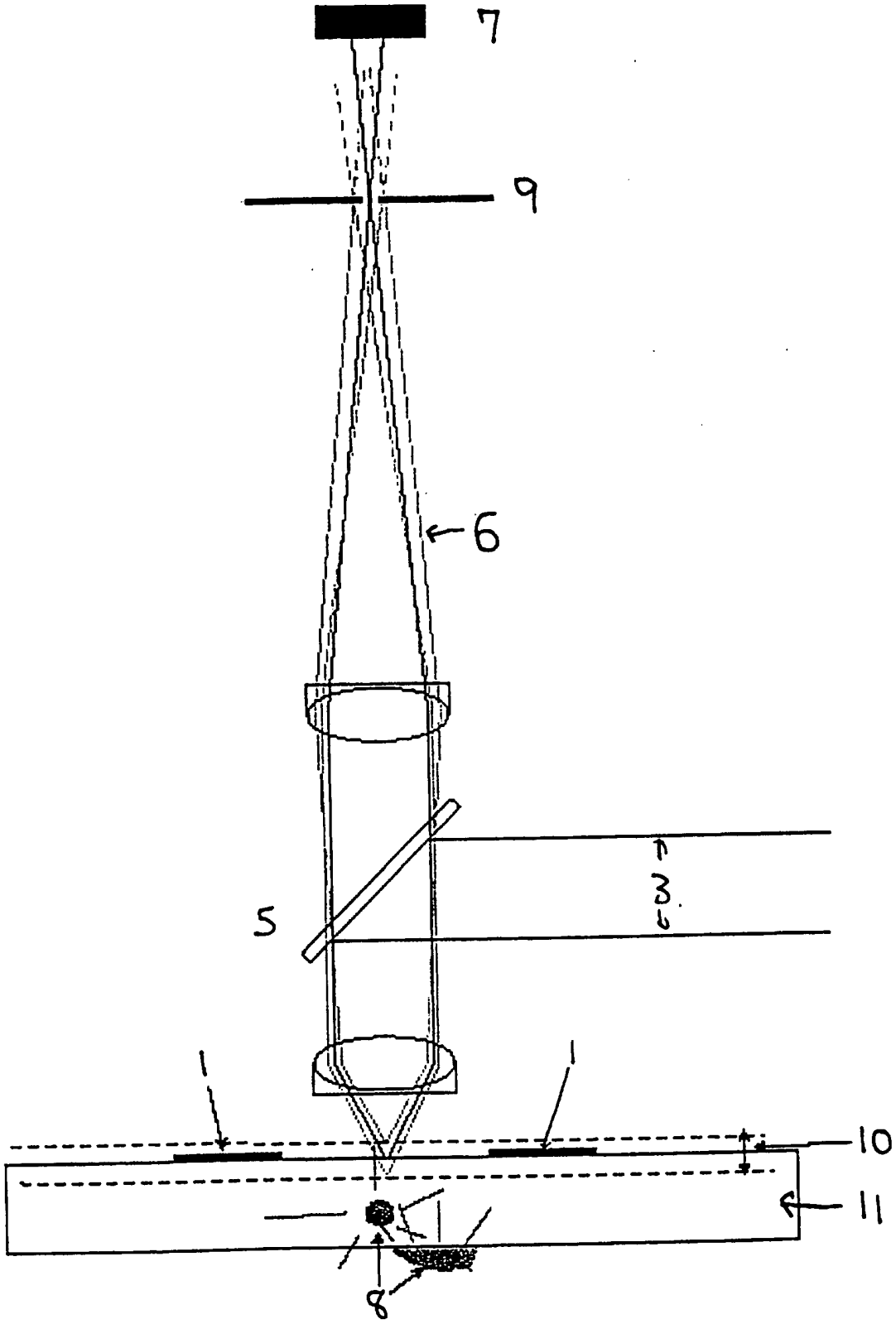


Figure 3.

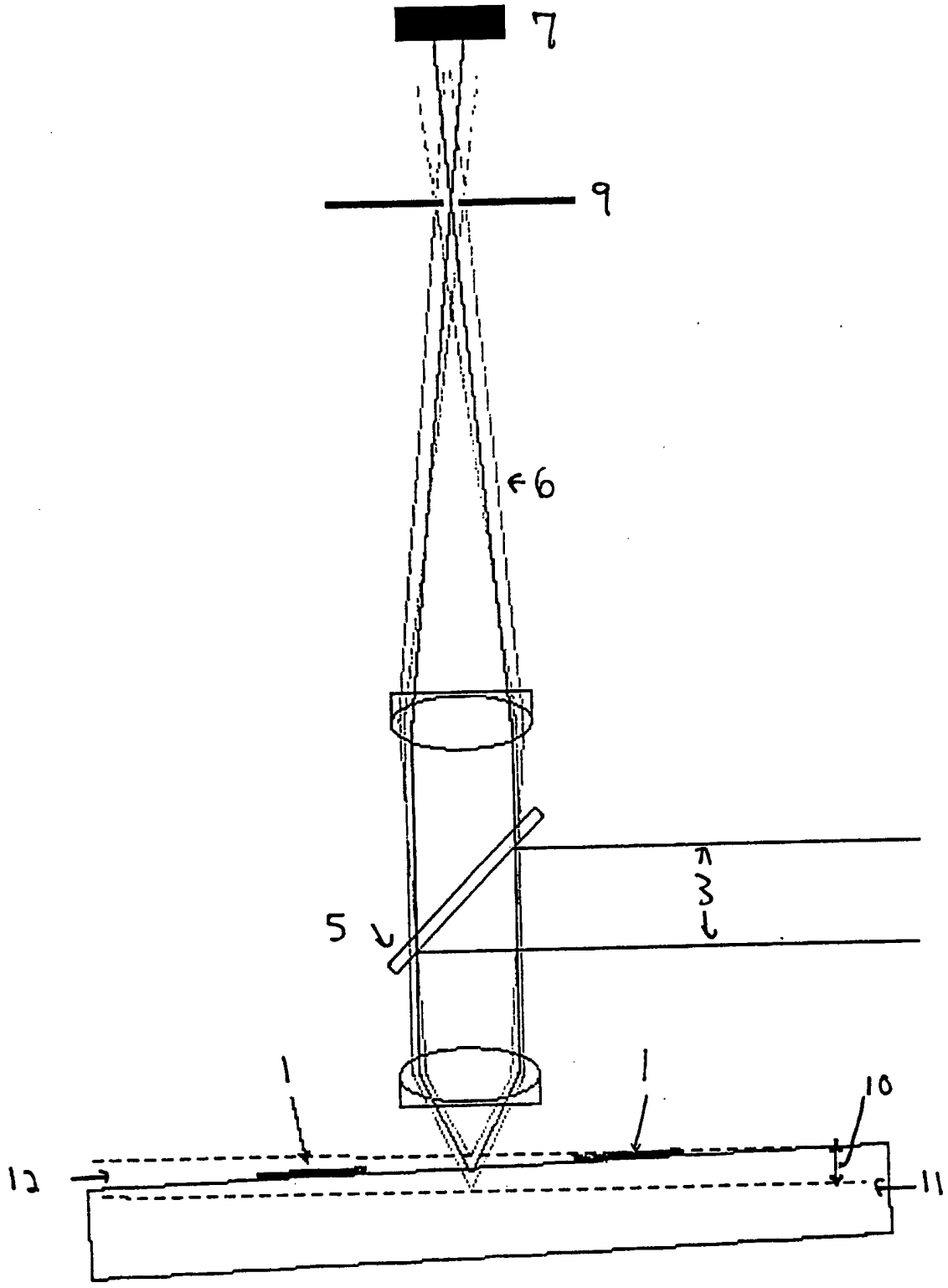
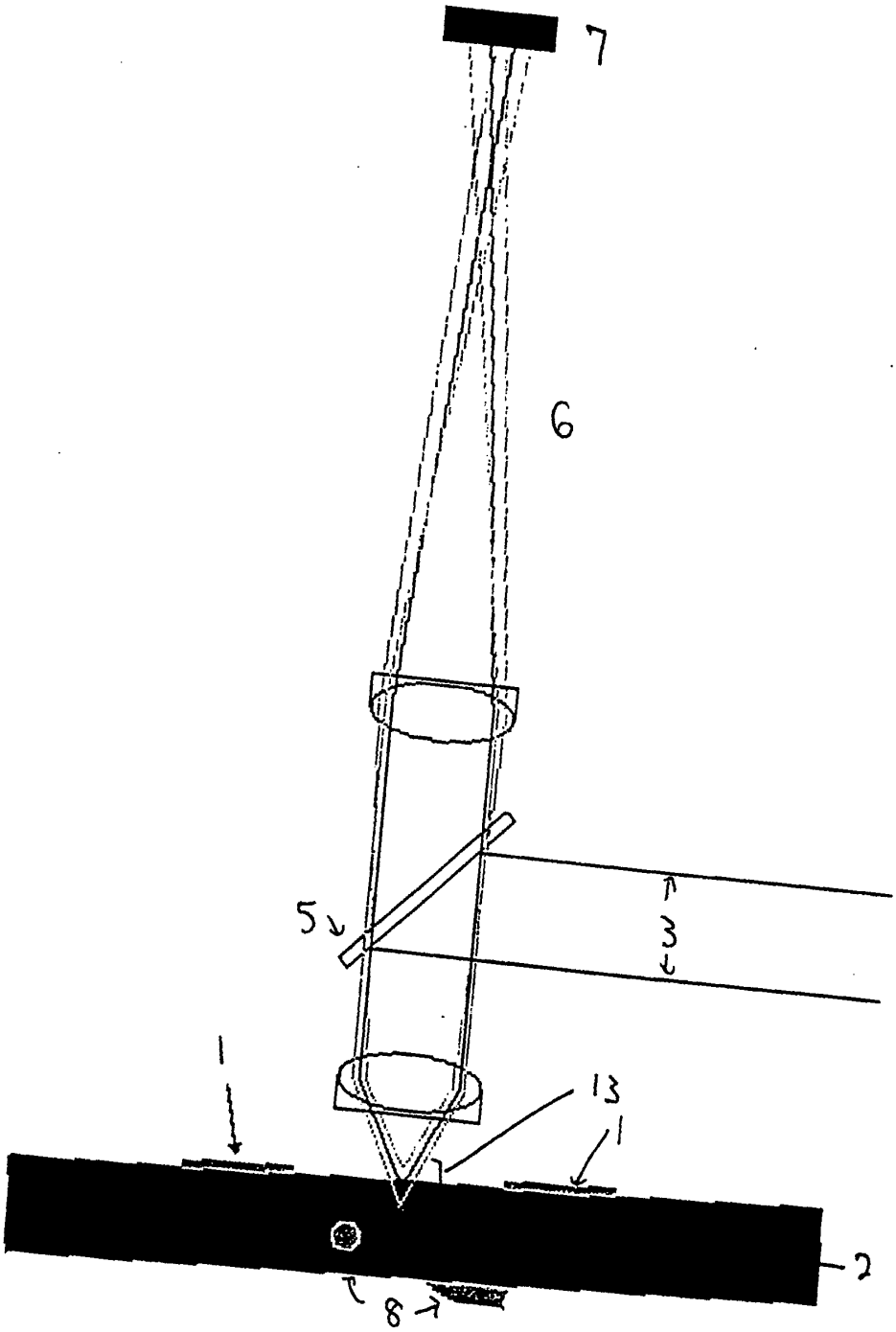


Figure 4.



Figur 5.

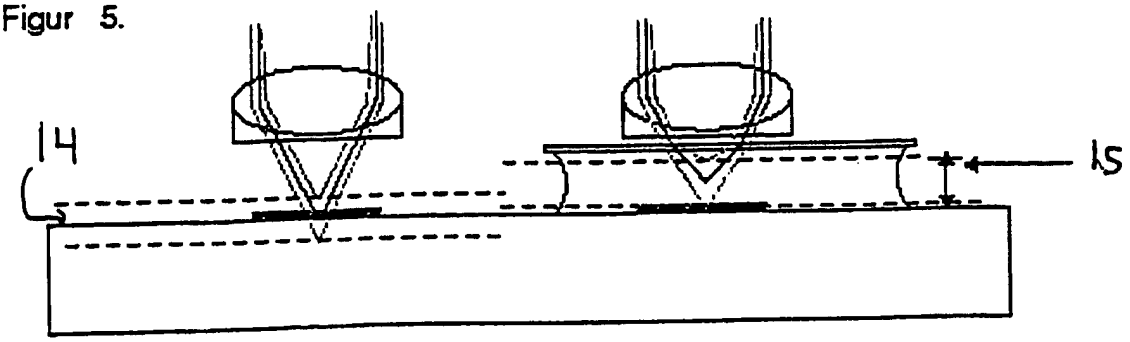


Figure 6.

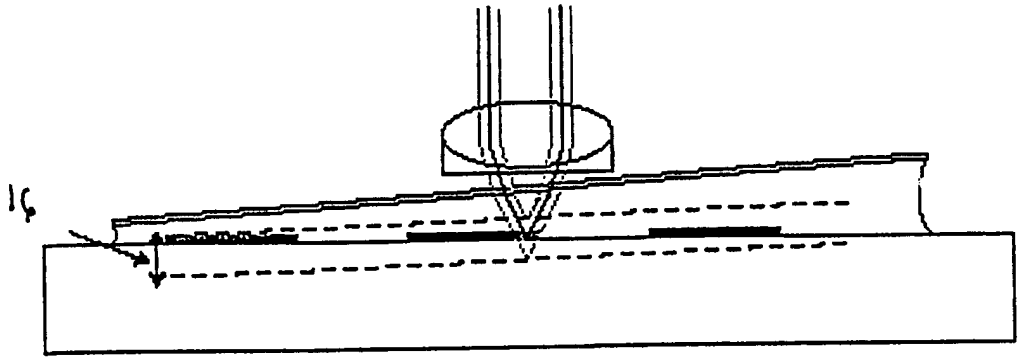
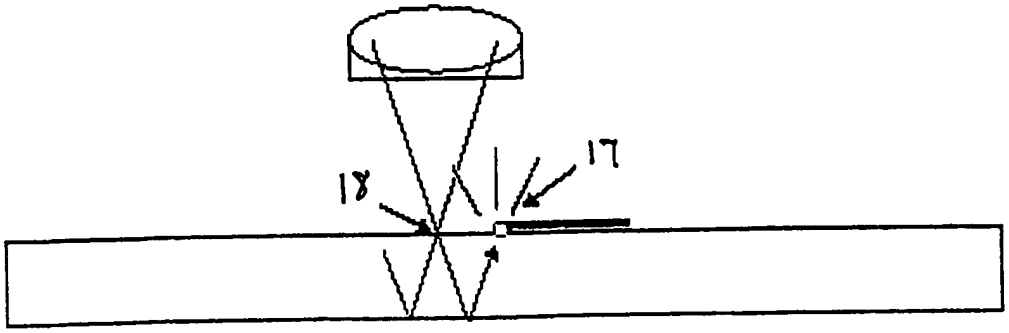


Figure 7.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28296

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G01N 21/64
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/58, 61, 63, 82.05, 82.08; 436/43, 46, 86, 93, 94, 172, 501, 518, 527

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - A	US 4,826,660 A (SMITH et al) 02 May 1989, see entire document.	27 ---- 1-26
Y	US 5,095,213 A (STRONGIN) 10 March 1992, see entire document.	1-27
Y	US 5,324,633 A (FODOR et al) 28 June 1994, see entire document.	1-27
Y	US 5,618,398 A (IZMAILOV et al) 08 April 1997, see entire document.	1-27
Y	US 5,639,671 A (BOGART et al) 17 June 1997, see entire document.	1-27
Y	WO 97/46597 A (BECKMAN INSTRUMENTS, INC.) 11 December 1997, see enitre document.	1-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28296

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,834,758 A (TRULSON et al) 10 November 1998, see entire document.	1-27
A	US 4,978,503 A (SHANKS et al) 18 December 1990.	1-27
A	US 5,568,400 A (STARK et al) 22 October 1996.	1-27

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

422/58, 61, 63, 82.05, 82.08; 436/43, 46, 86, 93, 94, 172, 501, 518, 527

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, CA and MEDLINE filed of STN and EAST
search terms: background, stray, auto, light, fluoresc?, fluorescence autofluoresc?, autofluorescence, reduc? reduce, reduction, reduced, elliminat?, elliminate, elliminated, ellimination, elliminating, eliminat?. eliminate. eliminated. eliminating, elimination, prevent?, prevent, prevented, preventing, prevention, attenuat?, attenuate, attenuated, attenuating, attenuation, minimiz?, minimize, minimized, minimizing, minimization, absorb?, absorb, absorbed, absorbing, absorption, substrate, plate, element, surface, support, neutral density, gray?, gray, grayed, grayish, dark?, darkened, black, blackened, colored, absorbing, antireflect?, nonreflect?, anti, non, low, relect?, antireflection, antireflecting, nonreflection, nonreflecting, reflection, reflecting, dna, rna, protein, peptide, polypeptide, oligonucl?, polynuclei?, polynucl$, bind, bound, binding, immobil$, microdot, micro dot, microarray, micro array, combinator?, array, glass